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Review

Microbial symbionts: a resource for the management of insect-related problems

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Summary

Microorganisms establish with their animal hosts close interactions. They are involved in many aspects of the host life, physiology and evolution, including nutrition, reproduction, immune homeostasis, defence and speciation. Thus, the manipulation and the exploitation the microbiota could result in important practical applications for the development of strategies for the management of insect-related problems. This approach, defined as 'Microbial Resource Management' (MRM), has been applied successfully in various environments and ecosystems, as wastewater treatments, prebiotics in humans, anaerobic digestion and so on. MRM foresees the proper management of the microbial resource present in a given ecosystem in order to solve practical problems through the use of microorganisms. In this review we present an interesting field for application for MRM concept, i.e. the microbial communities associated with arthropods and nematodes. Several examples related to this field of applications are presented. Insect microbiota can be manipulated: (i) to control insect pests for agriculture; (ii) to control pathogens transmitted by insects to humans, animals and plants; (iii) to protect beneficial insects from diseases and stresses. Besides, we prospect further studies aimed to verify, improve and apply MRM by using the insect–symbiont ecosystem as a model.

Introduction

Microbes and humans are strictly linked in every facet of the society (evolution, economy, behaviour and lifestyle). These interactions can bring about alternative effects from a human perspective. For instance, malaria (caused by *Plasmodium* parasites) is one of the major worldwide health emergences, and this disease represents a strong selective force on human populations. Indeed, in different malaria endemic areas, exposed populations developed genetic adaptations that confer resistance to the infection (Shi and Su, 2011). Moreover, the recent *Escherichia coli* outbreak in Germany (Nature Editorial, 2011, Vol. 474) underlined yet again how microbes can influence our life-determining public health emergencies even in developed countries (Fislage, 2011). On the contrary, there are several examples of beneficial interactions of microbes with plants, animals and humans, even in extreme conditions. For instance some bacteria are able to degrade contaminants and clean up polluted ecosystems (Balloi *et al.*, 2010), plant endophytes or rhizobacteria promote soil fertility and a safe plant growth even under environmental stresses (Hayat *et al.*, 2010), or animal gut symbionts are positively involved in the stimulation of the host's immune system and contribute to increase nutrient availability (Kinross *et al.*, 2011).

Although humans have unconsciously learnt to harness several microbial processes from the dawn of history, for
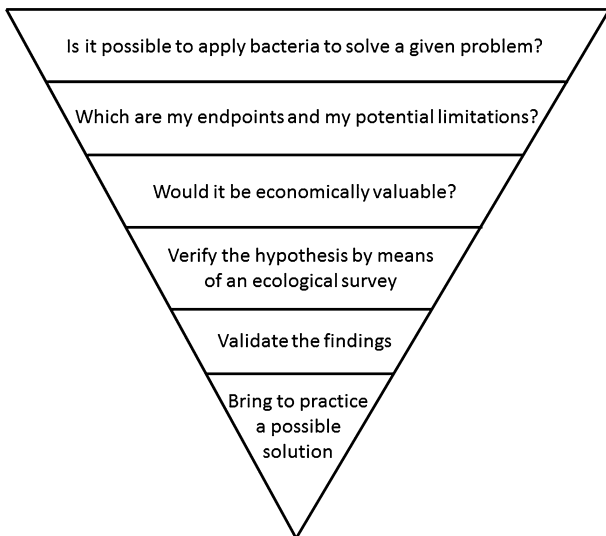
Fig. 1. MRM conceptual flow as adapted from Read and colleagues (2011).

example in the preparation of food (leavening of dough), beverage (fermentation of wine and beer) and tissues (soaking of linen), only from the second half of 1800 the development of microbiology slowly built up the awareness that it was possible to exploit the microbial metabolic capabilities for humans' benefit (Rittmann *et al.*, 2006). In 2007, Willy Verstraete theorized this concept and defined the Microbial Resource Management (MRM) as the human ability to manage complex microbial systems and their associated metabolic capabilities in order to solve practical problems (Verstraete, 2007). This led to the development of three parameters – Richness (Rr), Dynamics (Dy) and Functional organization (Fo) – to describe the complex microbial community and to answer questions like 'who is there?', 'who is doing what?', 'who is with whom?' (Marzorati *et al.*, 2008). This approach, originally designed for the ecological interpretation of raw fingerprinting patterns (e.g. DGGE, LH-PCR, t-RFLP), has been recently updated to be applied to the new molecular technologies (i.e. pyrosequencing), thus allowing to provide a more accurate picture of the complexity and variability of the microbial communities (Read *et al.*, 2011). Besides proposing a series of parameters to assess the 'nature' of a given microbial community, Read and colleagues (2011) also proposed a practical mind-set and a flow sheet based on the economical value of the approach, a clear determination of the end-points, and an ecological survey to determine the proper microbial weapons, in order to logically identify the correct direction to proceed when implementing the big picture of MRM (Fig. 1).

This new approach inaugurated a more conscious phase of the microbial ecology, no longer dominated by the inductive method and based on empirical observa-

tions, but by the application of microbial ecology theories, capable to explain and predict the behaviour of a given microbial community. The aim was to establish the base for the control and the steering of microbial resources.

A typical example is the change in prospective in the case of probiotics. At the beginning of the 20th century, Elia Metchinkoff, in his book *The Prolongation of Life*, hypothesized that the presence of lactic acid bacteria (LAB) in human intestinal tract could positively affect health and longevity. He based the hypothesis on the observation of the longevity of populations used to eat high amounts of yogurt (such as Baltic populations). Following this intuition, the concept of probiotic developed as the use of bacteria that could improve host health. However, the scientific literature presents many studies in which bacteria have been provided to humans with promising but often uncertain effects (Dunne *et al.*, 1999). Just to mention a few examples, the effect of an oral probiotic bacteriotherapy with *Lactobacillus rhamnosus* GG – previously shown to be effective in alleviating intestinal inflammation associated with food allergy in small children (Majamaa and Isolauri, 1997) – gave no beneficial effects once administrated to apple and birch-pollen-sensitive teenagers and young adults, who manifested intermittent symptoms of allergy and mild asthma (Helin *et al.*, 2002). The same LAB was shown to reduce the duration of viral diarrheal illness in European and North African children from 1 month to 3 years of age (Guandalini *et al.*, 2000), but not in Brazilian patients with similar traits (Costa-Ribeiro *et al.*, 2003). These and similar studies clearly show that the effectiveness of probiotics can be related to the patient traits, dietary habits (Hehemann *et al.*, 2010) and age (Biagi *et al.*, 2010) and that different people may have different needs. These examples show that, even if MRM was initially conceived as a practical approach for the development of an elaborative system that would describe and drive the management of the resources associated to a given microbial community, the practical implementation for many environments is still complex (Read *et al.*, 2011). This is mainly due to our limited understanding of those key factors that shape the composition and the activity of a microbial community in a complex environment.

Despite these limitations, there is a specific area in which MRM has been successfully applied nowadays. In fact, the recent literature in the entomological field (a simplified environment as compared with the human gut) can provide several examples in which the MRM concept has been used to practically solve real problems. The present work, after briefly discussing the biological role, sometime essential, of microbial simbionts in insects, aims to review these cases classifying them according to the purpose of the microbiota management: (i) for the control of insect pest for agriculture; (ii) for the control of insect-transmitted pathogens; (iii) for the protection of beneficial insects from

diseases and stresses. Moreover, this review will conclude analysing the possibility to develop future studies aimed to verify, improve and apply the MRM concept by using the insect–symbiont ecosystem as a model.

MRM of the insect microbiota

One of the environmental hot topics in MRM is the gastrointestinal tract (GIT), defined as an 'outside world inside the living animals' (Verstraete, 2007). The microbiota associated to the GIT is an highly complex community in which microbial cells outnumber, in the case of humans, prokaryotic cells by a factor of 10, comprising more than 1000 microbial taxa, most of which are unique to each host individual (Dethlefsen *et al.*, 2007; Ley *et al.*, 2008; Costello *et al.*, 2009; Qin *et al.*, 2010). This vast and diverse animal microbial ecosystem is a complex biological 'superorganism', whose components co-evolved with the host, and play an essential role for the host's health and the metabolic regulation. With regards to the invertebrate gut, the microbial communities are generally less complex if compared with those of mammals, with one or two orders of magnitude less in terms of richness. However, remarkable differences could be found among species (Dillon and Dillon, 2004; Dunn and Stabb, 2005; Behar *et al.*, 2008; Hongoh, 2010; Robinson *et al.*, 2010; Wong *et al.*, 2011). For instance, termite's microbiota is more complex than fruit fly's one. In fact, the former harbours several hundred species of gut microbes unique to termites, comprising protists, bacteria and archea (Hongoh, 2010), while the fruit fly *Drosophila melanogaster* less than 10 (Wong *et al.*, 2011). Despite these differences microbes exert important and crucial functions for the survival and benefit of the host also in insects. In particular, the interactions established between bacteria and insects, or arthropods in general, have been known since long to go beyond pathogenesis (Dale and Moran, 2006).

Cellular and humoral defences are deployed by insects to defend themselves from pathogens and parasites. Inherited protective microbes act as an additional exogenous immune system, highlighting their great relevance in preserving insect health (Hurst and Hutchence, 2010). Commensal bacteria can modulate the innate immune system and strengthen the epithelial barrier, limiting pathogenic bacterial contact with the epithelium by inducing the secretion of antimicrobial compounds or competing with them (Hamdi *et al.*, 2011). For instance, in the case of aphids, we can find several examples of symbiont-mediated protection. Besides the obligate mutualistic symbiont *Buchnera aphidicola*, the aphid *Acyrthosiphon pisum* harbours one or more facultative symbionts, i.e. *Hamiltonella defensa*, *Regiella insecticola* and *Serratia symbiotica.* They explicate a role of protection of the aphid against natural enemies, such as entomopathogenic fungi

and parasitoid wasps, or against heat stress (Oliver *et al.*, 2010). Also *Drosophila* in nature is commonly defended by protective symbionts. *Wolbachia* infection in the fruit fly results in a strong resistance to RNA virus infection (Hedges *et al.*, 2008; Teixeira *et al.*, 2008).

To exploit gut microbes in a MRM approach, firstly, the healthy intestinal microbiome must be understood, in terms of diversity and functionality. The diversity of the gut microbiota is linked to the genotype, diet, developmental stage, sex and physiological conditions of the host (Dethlefsen *et al.*, 2007; Sharon *et al.*, 2010). In the case of *Drosophila melanogaster,* it has been shown that the gut microbiome was constituted by *Lactobacillus, Enterococcus* and *Acetobacter* members, in several studies performed on the same species by different authors (Corby-Harris *et al.*, 2007; Cox and Gilmore, 2007; Ren *et al.*, 2007; Ryu *et al.*, 2008; Crotti *et al.*, 2010). This is in analogy with the human gut in which recently it has been identified a 'core' microbiome (Turnbaugh *et al.*, 2009). Studies performed on honeybees collected from different geographic regions, such as South Africa (Jeyaprakash *et al.*, 2003), Germany (Mohr and Tebbe, 2006) and Switzerland (Babendreier *et al.*, 2007), gave a similar picture: the presence of a core bacterial microbiota conserved worldwide (Hamdi *et al.*, 2011). On the other side, in the case of the cabbage white butterfly, the bacterial community shows temporal instability at the species level and conservation at phylum level (Robinson *et al.*, 2010). These examples show how in different species, nature apparently selected for different mechanisms of adaptation. The essential factor is to maintain the overall functionality of a community rather than to conserve the presence of particular members (Robinson *et al.*, 2010). Cases in which the gut functionality is disrupted by specific changes in the composition of the resident microbial community are known as dysbiosis. This is often referred to as a perturbation of the intestinal microbe–host homeostasis and it can be implicated with a pathological state, explicating a role in the occurrence of a disease. An example of insect dysbiosis has been reported by Cox-Foster and colleagues (2007). By the use of a metagenomic survey, it has been demonstrated that in the microbiota of healthy bees there is a predominance of *Alphaproteobacteria* and *Firmicutes*, which are not found when bee specimens affected by colony collapse disorder (CCD) are analysed. A phenomenon of dysbiosis occurs in this case and the restoration of a healthy microbiota could counteract the microbial disequilibrium. In humans, such conditions are normally treated by means of therapeutic approaches – such as bacteriotherapy (Borody *et al.*, 2004) and bioecological control (Bengmark, 2005) – which make use of pre- and probiotics (or a combination of the two – 'synbiotics') in order to modulate the intestinal microbial community and improve the human health. In

the next paragraph we will evaluate how this modulation can be translated in the insect world.

Symbiont management in insect pests for agriculture

An elegant example of the manipulation of the insect microbiota is the management of the bacterial community associated to the Mediterranean fruit fly, *Ceratitis capitata* (Ben Ami *et al.*, 2010; Gavriel *et al.*, 2011). One of the strategies, commonly used to control this invasive pest, is the sterile insect technique (SIT) that foresees, firstly, a mass rearing of overwhelming numbers of male individuals, followed by insect sterilization by gamma irradiation and finally their release in the target area. After releasing, the sterile males compete with the native males for the mating with wild females and, in a successful scenario, the reduction of the next fly generation is expected. However, several studies have emphasized that irradiated males are less competent in attracting and mating with wild females than wild males. As demonstrated by molecular tools by Ben Ami and colleagues (2010), gamma irradiation influences the fly's gut microbial community leading to a dramatic reduction of *Klebsiella* sp. and to a problematic increase of *Pseudomonas* sp. Therefore, a clear case of dysbiosis due to the irradiation process affects phenotypically the sterile male performances. In order to restore the original microbial community, Ben Ami and colleagues (2010) fed the insects with the fly symbiont *Klebsiella oxytoca*. The administration of *K. oxytoca* led to its stable colonization and a decrease of potentially pathogenic *Pseudomonas* spp., resulting in a higher mating competitiveness as compared with wild males. Furthermore, other experiments performed on captured wild medflies had showed that the administration of high levels of a mix of bacteria belonging to the *Enterobacteriaceae* family – previously isolated from the fly community – and in which one of the members was *K. oxytoca*, extended the fly's longevity (Behar *et al.*, 2008). This approach could be applied in order to extend the life span of sterile male insect and to enhance the success of SIT programs. The reported examples show that the manipulation of the insect microbiota by the administration of members of the fly's community can positively influences several aspects of the insect life. In MRM terms, these experiments showed that within a plan of biological control strategy against a pest, it is of key importance to consider the role of the whole microbiota of the target insect. In the three mentioned studies (Behar *et al.*, 2008; Ben Ami *et al.*, 2010; Gavriel *et al.*, 2011), the authors were able to reach successful results by applying an MRM approach: they use molecular tools in order to: (i) evaluating the microbial community structure, satisfying the question 'who is there'; (ii) defining the key microorganisms, satisfying the question 'who is doing what'; and (iii)

planning the strategy to restore the suitable climax community, satisfying the question 'who is with whom'.

Another strategy proposed for the control of *C. capitata* foresees the use of cytoplasmic incompatibility (CI)-inducing *Wolbachia* endosymbionts as a novel environmental-friendly tool (Zabalou *et al.*, 2004). *Ceratitis capitata* is generally not infected by *Wolbachia*, although a few records referred to the presence of this symbiont in some Brazilian medflies (Rocha *et al.*, 2005; Coscrato *et al.*, 2009). *Wolbachia* transinfections from a closely related species of the medfly, *Rhagoletis cerasi*, allow obtaining *Wolbachia*-transinfected lines of *C. capitata*, stably infected with the bacterium with rates of 100% and able to express the CI phenotype. Results obtained by Zabalou and colleagues (2004) evidenced that for the suppression of the insect pest a release of *Wolbachia*-infected medflies could be successfully and efficiently used, as demonstrated by laboratory cage trials. This study is an example of a more general application of *Wolbachia* or of other CI-inducing agents in strategies defined 'Incompatible Insect Technique' (IIT). The introduction of *Wolbachia* into pest and vector species of economic and hygienic relevance could be a powerful tool to suppress or modify natural populations. For a successful implementation of IIT it is mandatory to employ an efficient sexing strain of the insect pest, in order to release only the males. Thus, a medfly line infected with CI-inducing *Wolbachia* and carrying the selectable marker *temperature sensitive lethal* (*tsl*) for the male-only production has been developed by Zabalou and colleagues (2009).

Insect mass rearing for SIT is widespread all over the world. In 2002, it has been estimated that more than 1.4 billion sterile male-only pupae were produced per week in different facilities around the world. The SIT programs contributed to the eradication of some insect species from specific regions, such as the New World Screwworm eradicated from Libya or the tsetse fly from Zanzibar (Lindquist *et al.*, 1992; Reichard, 2002). The sterile insect technique is applied on different insect species and its economic and social benefits have been demonstrated in various cases (Vargas-Terán *et al.*, 2005). The process of implementing SIT requires seven components: suppression of density, mass rearing, sterilization, shipment, release, evaluation, and quality control.

The application of this MRM approach for SIT or IIT could contribute to the implementation of these techniques for the production of males more competitive than wild ones or with *Wolbachia*-induced CI trait for other species of insect.

A microbial tool widely used in biocontrol programs of specific insect species is represented by the use of the entomopathogenic bacterium *Bacillus thuringiensis* (Bt). Bt has been widely studied for its ability to produce parasporal crystalline protein inclusions, usually indicated as crystals, which explicate interesting and exploitable

insecticidal activities. Bt ability has been used worldwide for the biocontrol of insect pests and for the development of transgenic crops (van Frankenhuyzen, 2009). Recently, the '*B. thuringiensis* toxin specificity database' has been designed to collect information on the biological specificity of the individual crystal proteins available in literature (K. van Frankenhuyzen and C. Nystrom, http://www.glfc.forestry.ca/bacillus, January 2008; van Frankenhuyzen, 2009). Nowadays, Bt has become the leading biological insecticide and, along with *Bacillus sphaericus,* it has also been successfully used to control the mosquito vectors of diseases, such as dengue and malaria (Becker, 2000). The use of biopesticides as a component of integrated pest management (IPM) have been gaining acceptance over the world. However, in some cases, the lack of proper strategy and effective application methods are among the reasons why the usage of Bt is not successful, as it has been recorded for Bt ssp. israelensis in Malaysia (Lee *et al.*, 2006). The application of the MRM mind-set in this field could enhance the exploitation of this microbial insecticide, which has proven to possess interesting features such as the safety for non-target organisms, high specificity, easy productivity of the commercial formulates and realistic market positioning.

Symbiont management in insect vectors to control the carried pathogens

Still nowadays infectious diseases pose real and several problems, especially in developing countries, with diseases like malaria, trypanosomiasis, lymphatic filariasis and onchocerciasis, which are vectored by arthropods. In order to eliminate or block the diffusion of a pathogen, one of the recently proposed strategies is based on the exploitation of mutualistic symbiotic bacteria, which are associated to the host vector or to the pathogenic agent and which are essential for the host survival or pathogen reproduction. In this respect, they can be considered as the final target for 'chemotherapy treatments'.

An explicative example is again on the *Alphaproteobacterium Wolbachia*. Generally, *Wolbachia* is not a primary symbiont since it is not essential for the insect survival, though exceptions have been found, like in the case of the *Drosophila* parasitoid, *Asobara tabida*, where *Wolbachia* is necessary for the wasp oogenesis (Dedeine *et al.*, 2001). On the other hand, in nematodes as *Brugia malayi*, *Wuchereria bancrofti* and *Oncocherca volvulus* (agents of lymphatic filariasis and river blindness) *Wolbachia* is a primary obligate symbiont, essential for the host development and survival. The principle of treating filarial diseases through antibiotic treatment exploits this strict association with the host. The therapeutic approach has been attested by multiple studies in which the anti-filarial effects of antibiotics such as doxycycline or rifampicin on

nematodes have been evaluated in laboratory conditions and by several clinical trials in humans (Bandi *et al.*, 1998; 1999; Taylor *et al.*, 2005; Bazzocchi *et al.*, 2008; Hoerauf, 2008; Supali *et al.*, 2008; Coulibaly *et al.*, 2009; Mand *et al.*, 2009; Specht *et al.*, 2009; Wanji *et al.*, 2009). Nowadays, mass drug administration (MDA) is used worldwide for the elimination of filariasis, but the employed drugs only temporarily clear the juvenile stage of nematodes without killing all adult specimens (Gyapong *et al.*, 2005). The antibiotic-based treatments against *Wolbachia* are among the top research priorities with new promising insights. The Anti-*Wolbachia* Consortium, A-WOL, was thus established with the aim to discover and develop new anti-*Wolbachia* drugs and application, with therapies compatible with MDA (Taylor *et al.*, 2010). This is a clear example of how the manipulation of the host microbiota, with the elimination of an essential primary endosymbiont, results in the impairing of a highly virulent and pathogenic parasite.

Essential for the transmission of a pathogen is that the pathogen spends a period of extrinsic incubation into the vector, in order to be transmitted. This means that only the vectors from a defined age are able to transmit the pathogen, that is to say that only the oldest part of the vector population transmit the pathogen. *Wolbachia* strain *w*MelPop, a symbiont of *Drosophila*, is a life-shortening strain, therefore able to reduce adult life span of its natural host and, as a consequence, to reduce pathogen transmission (McMeniman *et al.*, 2009). A recent strategy proposes to transfer this strain in vectors of medical and agriculture importance. In order to get this achievement in mosquito-transmitted diseases, scientists firstly adapted *w*MelPop from *Drosophila* in a mosquito cell culture for 3 years and then they microinjected the adapted *w*MelPop strain into naturally uninfected embryos of the major mosquito vector of dengue *Aedes aegypti*. Strain *w*MelPop halved the life span of the mosquito, inducing CI and maintaining high maternal inheritance, with no differences in fecundity (McMeniman *et al.*, 2009). *Wolbachia* is a powerful tool for the control of vector-borne diseases. In this standpoint different scenario can be pictured: (i) *Wolbachia* can be used as a 'gene driven agent', able to 'drive' refractory genes into the vector population (Rasgon *et al.*, 2006); (ii) *Wolbachia*-infected males can be released into the insect population and, through *Wolbachia*-induced CI, it could be obtained a reduction of vector population (see previous paragraph); (iii) insect vectors with virulent or pathogenic strains of *Wolbachia* can be released, as the case of the aforementioned *w*MelPop strain, able to shorter the host life span (McMeniman *et al.*, 2009). Moreover, it has been observed that *Wolbachia* is able to exert an interference with transmitted pathogens, being able to inhibit *Plasmodium falciparum* oocsts in mosquito midgut, or the devel-

opment of the infectious stage of filarial nematodes (Kambris *et al.*, 2009; Hughes *et al.*, 2011).

Formulations based on entomopathogenic fungi have been proposed as powerful tools in the control of vector-borne diseases. *Metarhizium anisopliae* and *Beauveria bassiana* have been shown to efficiently infect and kill mosquito larvae in laboratory trials (Scholte *et al.*, 2005). Also recombinant strains of *M. anisopliae*, expressing molecules whose targets were *Plasmodium* sporozoites, in a variation of the so called 'paratransgenesis approach', resulted in a high inhibition of the malaria protozoan (Fang *et al.*, 2011). Specific formulations have been developed in order to prepare a more useful and persistent product under field conditions for the control of malaria-transmitting anophelines (Bukhari *et al.*, 2011). It is not only important to evaluate the effective agent for the foreseen application, but also to consider the best carrier for the delivery of a product and the best delivery way (where, when and how) in order to scale up the procedure from the laboratory condition to the open field.

Paratransgenesis was firstly introduced with the study carried out on the triatomine *Rhodnius prolixus*, the vector of the parasitic protozoan *Trypanosoma cruzi*, the causative agent of the Chagas disease (Beard *et al.*, 2001). A member of its microbial community, *Rhodococcus rhodnii*, essential for the growth and development of the host, has been genetically modified (GM) to express trypanocidal genes and then it has been 're-introduced' into the host. A formulation based on GM bacteria, named CRUZIGARD, has been developed, at a laboratory scale, in order to introduce GM symbionts into its host, resulting in a successful application method.

Similarly, in the tsetse fly *Glossina morsitans*, vector of *Trypanosoma brucei*, the etiological agent of the sleeping sickness, its secondary symbiont *Sodalis* has been proposed as a paratransgenic tool to block the transmission of the disease. *Sodalis* shows a wide tropism in the tsetse body, being mainly localized at the midgut level (Rio *et al.*, 2004) and within the cytoplasm of the secretory cells (Attardo *et al.*, 2008).

Promising tools in the control of disease-transmitting mosquitoes like *Anopheles* are the acetic acid bacterial symbionts of the genus *Asaia* (Favia *et al.*, 2007; Crotti *et al.*, 2010). *Asaia* is tightly associated to different organs and tissues of the *Anopheles* body, including salivary glands and midgut that represent 'key spots' for the development and the transmission of the malarial pathogens. Moreover, several features of *Asaia* account for making it a powerful instrument in a applications of MRM applied to the insect microbiome: (i) the high prevalence and relative abundance in the mosquito individuals and populations (Favia *et al.*, 2007; Chouaia *et al.*, 2010); (ii) the versatility to be transmitted by horizontal (via co-feeding or venereal) and vertical routes (maternal or paternal; Damiani *et al.*,

2008; Crotti *et al.*, 2009; Gonella *et al.*, 2011); (iii) the ability to efficiently spread through insects populations supported by the capacity of the bacterium to colonize and cross-colonize phylogenetically related or distant hosts (Crotti *et al.*, 2009); and (iv) the ease to be transformable with exogenous DNA (Favia *et al.*, 2007; Crotti *et al.*, 2009).

Similarly, very recently it has been proposed another symbiont of *Anopheles*, the *Gammaproteobacterium Pantoea agglomerans* as a potential carrier of antagonistic factors against *Plasmodium* (Riehle *et al.*, 2007). By using suitable heterologous secretion signals several anti-*Plasmodium* effector proteins could be efficiently secreted by the strain without apparently affecting the growth rate in the mosquito midgut (Bisi and Lampe, 2011).

Another microorganism with a potential for the control of mosquito-borne diseases is the *Saccharomycetales* yeast, *Wickerhamomyces anomalus,* previously known with the name of *Pichia anomala* (Ricci *et al.*, 2011a,b). *Wickerhamomyces anomalus* has been identified in several *Anopheles* and *Aedes* species as a stably associated symbiont in the host midgut and reproductive systems. Great attention is placed towards the use of a paratransgenesis approach based on genetically modified yeasts that, as eukaryotic organisms, could allow solving translation and folding biases of eukaryotic recombinant proteins.

Insect-transmitted plant pathogens are another area in which the MRM approach could be applied with success. More precisely, research has been conducted on phytoplasmas, vectored by leafhoppers, *Liberibacter* pathogens transmitted by psyllids, and the *Gammaproteobacterium Xylella fastidiosa,* spread by the glassy-winged sharpshooter *Homalodisca vitripennis.* All these microorganisms are responsible of plant diseases that cause devastating yield losses in diverse low- and high-value crops worldwide. Disease control is commonly based on the control of the insects, i.e. by spraying various insecticides, and on practices that consist in the removal of symptomatic plants. However, some first steps of MRM applications have been already carried out on the vectors, with the aim of defining the microbial community composition and functionality in the insects (Marzorati *et al.*, 2006; Miller *et al.*, 2006; Crotti *et al.*, 2009; Raddadi *et al.*, 2011). The final aim is to propose a biocontrol approach based on the management of the microbial symbionts associated to the vectors in order to counteract directly the pathogen or to reduce the vector competence.

An example is represented by the Pierce's disease of grape caused by the above mentioned *X. fastidiosa*. A culturable bacterial symbiont of the *X. fastidiosa* vector *H. vitripennis* has been isolated from the host foregut. This symbiont, identified as an *Alcaligenes xylosoxidans* ssp. *denitrificans*, was capable of colonizing the same niche, the foregut, occupied by *X. fastidiosa* indicating that it has

the basic potential of counteracting the pathogen for instance by competitive exclusion during the colonization of the host foregut. By using a variant of the strain transformed with a plasmid for the expression of a fluorescent protein, it was possible to track the behaviour of the symbiont within the host body. A characteristic potentially very useful for the development of an approach of symbiotic control of the Pierce's disease is the versatility of the strain in colonizing different host type. It has been shown that the specific strain of *A. xylosoxidans* ssp. *denitrificans* is capable to behave as a plant endophyte in grape. Such a feature could be positively exploited to increase the exposure of the transmitted pathogen to antagonistic factors expressed by the bacterial symbiont not only at the level of the insect body but in the target plant species too (Bextine *et al.*, 2004; Bextine *et al.*, 2005; Miller, 2011).

Symbiont management in the protection of beneficial insects

When people think to insects, or arthropods in general, they have the idea of 'pests' or 'disease vectors'. However, most of the insects are useful for human and environmental benefit. Some of them (bees, wasps, butterflies and ants) are pollinators, others reduce the population of harmful insects, representing a real alternative to chemical application. Others produce useful substances for human activities, as honey, wax, lacquer and silk. Lastly, in many countries, insects are a part of people's diets and edible insects, such as caterpillars and grubs, are important sources of protein.

Nowadays, a serious environmental problem is the decline of pollinators and a number of firms are working in the perspective of producing insect species for pollination management in the field, orchards and greenhouses at the flowering time. Honeybees and bumble-bees are sold worldwide and guidelines and operative protocols are provided to farmers for an optimal application. However, these beneficial insects are coping with severe stresses, including both abiotic and biotic ones (e.g. parasites, fungi, bacteria and viruses), which are seriously affecting their wellness, activity and productivity. Management of microbial symbionts could represent a mean to enhance the defences of beneficial insects from pathogens' attacks. Some microbial groups, as LAB or acetic acid bacteria (AAB), have been reported as able to enhance innate immune system of bees or fruit flies (Evans and Lopez, 2004; Ryu *et al.*, 2008). Indeed, LAB and AAB are generating a lot of interest in apiculture, the former for the potential probiotic activity, the latter because it has been shown to be abundant and prevalent symbionts in healthy insects with sugar-based diets (Crotti *et al.*, 2010). LAB and AAB own specific features that make them efficient colonizers of the bee midgut in comparison to acid-

sensitive pathogens. For instance they are able to tolerate low pH, to produce organic acids and to utilize a wide range of sugars, interfering with the potential establishment of pathogenic bacteria. Other commensals of the honey bee gut like those of *Bacillus* and related genera have been recently shown to have an antagonistic effect against *Paenibacillus larvae*, the causative agent of American Foulbrood disease (AFB, Cherif *et al.*, 2008; Hamdi *et al.*, 2011). In general, we can say that this could open the possibility – in MRM terms – of acting on the microbial structure and functionality of a specific niche in order to re-establish a good balance of the microbiota with a benefit for the host.

Recently, by using artificial microcosms, it has been proved that microorganisms, once present in a suitable climax community, guarantee a high functionality of the system even during stressing events (Wittebolle *et al.*, 2009). In the case of the gut microbiota, this functionality contributes to the host protection against pathogen infections (see the review of Hamdi *et al.*, 2011). In particular in a recent work, it was demonstrated that structural changes in the midgut bacterial communities of cabbage white butterfly (*Pieris rapae*) larvae, due to variations in the diet, enhanced the susceptibility to biological invasion. Two different experiments were conducted. In the first trial, the community of a pool of larvae fed with an artificial diet was compared with other two pools of larvae fed with the same diet, but enriched with Brussels sprouts or sinigrin respectively (both exert an anti-microbial activity). In the second trial, larvae were fed with a sterile artificial diet both in the presence and in absence of antibiotics. Subsequently, the larvae were exposed to bacteria, commonly present within the larval microbiota, but exogenous to the diet. At the end of the treatment, the microbial community of all the larvae was characterized by using 16S rRNA gene clonal library technique. The study revealed that, compared with the microbiota of the larvae reared with the sterile artificial diet, those exposed to antibiotics, Brussels sprouts and sinigrin were altered in their structure, resulting to be more susceptible to the invasion (Robinson *et al.*, 2010).

This study, which provides clear evidences on the importance of the native community structure in preventing exogenous invasions, results in particular interest when the MRM parameters are applied to describe the degree of the perturbation of the microbiota organization in the different treatments. Of particular utility are the Ecological Pareto value (Ep), which describes the optimal microbial community organization for a specific environment, and the Community distortion factor (Cd) that calculates the degree to which the Community organization (Co) is different from the Ecological Pareto value (Read *et al.*, 2011). In both proposed experiments we can consider as the EP value the one referred to the structure of the microbiota of the control community (sterile diet) and

as Co the value of the microbiota subjected to changes in the diet. In both experiments, the Cd factors resulted in a value different from the one of EP, indicating that the communities have a low resistance to the applied perturbations (Co values were -24.04, -24.03 and -33.72 for the communities of the larvae fed with sinigrin, Brussels sprouts and antibiotics respectively).

These results numerically support the observation that perturbations can decrease the resistance of the communities to invasion.

Future perspectives

In this review, we have evaluated the different possibilities in which the manipulation of the microbial community associated to the insects can be carried out in order to obtain multiple benefits. However, this is just the 'top of the iceberg' and many other possibilities lay in the future. The influence of the microbial partners on the biology and evolution of a eukaryotic host is nowadays well recognized but the main drivers are frequently unknown. This can be highly appreciated in relation to the 'hologenome theory of evolution' (Rosenberg and Zilber-Rosenberg, 2011). This theory considers the holobiont (the host organism and its symbiotic microbiota) with its hologenome (the sum of the genetic information of the host and its microbiota) acting in a consortium as a dynamic entity and a unit of selection in which some microorganisms multiply and other decrease in number as a function of local condition within the holobiont (Rosenberg and Zilber-Rosenberg, 2011). Due to such a close relationship, the possibility of managing the microbial community opens several perspectives in terms of MRM in relation to the comprehensive characterization of the microbiota and the determination of its role in health and disease. The understanding of these principles and the definition of general ecological rules are of key importance to implement MRM to practice. For instance, this is the aim of the Human Microbiome Project that has been initiated by the NIH Roadmap (http://nihroadmap.nih.gov/hmp/). However, mammals are far too complex for basic ecological studies. On the contrary, this is not the case for insects that, in comparison to humans, are a more simplified system. This leads to a double opportunity for the insects. On the one side, due to their relatively easy growth under controlled conditions, the possibility to manipulate both hosts and symbionts, the ability to determine precisely the kind of interactions between the partners and the possibility to measure the effects of these interactions, insects can be a more handy holobiont to study specific theories of microbial ecology and develop new aspects of MRM approach. On the other side, extra work has to be conducted to further exploit the MRM approach in the insect world. For example, the already developed MRM param-

eters (Marzorati *et al.*, 2008; Read *et al.*, 2011) do not take in consideration the role of the communication occurring among cells within the microbiota and between cells host and microbiota. The cellular communicative strategies, inter- and intra-taxa, are quite complex, comprising conjugation systems, secretory systems, systems that use small hormone-like signalling molecules, plasmodesmatas, gap junctions and tunnelling nanotubes and probably other still unknown mechanisms (Dubey and Ben-Yehuda, 2011). This ecological aspect can be a promising field of application of MRM to control and manage the ecosystem symbiont-insect.

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